Detection of Forensically Relevant Nitazenes Using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

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INTRODUCTION

Synthetic opioids contribute to many of the overdose deaths in the United States. This has included structurally distinct classes of novel synthetic opioids, such as the nitazenes, particularly since the scheduling of the core structure of fentanyl [1]. The number of forensically relevant nitazenes has continued to increase and the most prevalent analogs are constantly fluctuating. Toxicology laboratories must constantly develop or adapt methods to detect the most relevant compounds with sufficient selectivity and sensitivity. Hence, the goal of this project was to develop and validate a method for the quantitation of seven forensically relevant nitazenes in whole blood using LC-MS/MS (**Figure 1 and Table 1**).

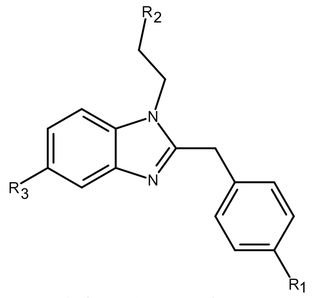

Figure 1. *Core structure of nitazenes*

Table 1. *Structural modifications of nitazenes included in this method*

Compound	R1	R2	R3
Isotonitazene	$(CH_3)_2CHO$	$(CH_3CH_2)_2N$	NO_2
Metodesnitazene	CH_3O	$(CH_3CH_2)_2N$	H
N-pyrrolidino etonitazene	CH_3CH_2O	$(C_4H_8)N$ (ring)	NO_2
Protonitazene	$CH_3CH_2CH_2O$	$(CH_3CH_2)_2N$	NO_2
N-piperidinyl etonitazene	CH_3CH_2O	$(C_5H_{10})N$ (ring)	NO_2
5-methyl etodesnitazene	CH_3CH_2O	$(CH_3CH_2)_2N$	CH_3
4-OH nitazene	OH	$(CH_3CH_2)_2N$	NO_2

REFERENCES

1. Papsun, D.M., Krotulski, A.J. and Logan, B.K. (2022) Proliferation of novel synthetic opioids in postmortem investigations after core-structure scheduling for fentanyl-related substances. The American Journal of Forensic Medicine and Pathology, 43, 315–327.
2. Krotulski, A.J., Walton, S.E., Fogarty, M.F., Papsun, D.M. and Logan, B.K. (2021) Metonitazene NPS toolkit.
3. ASB/ANSI (2019) ASB standard 036: Standard practices for method validation in forensic toxicology.

DISCLOSURE

The authors do not have any conflicts of interest to disclose.

ACKNOWLEDGEMENTS

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RESULTS & DISCUSSION

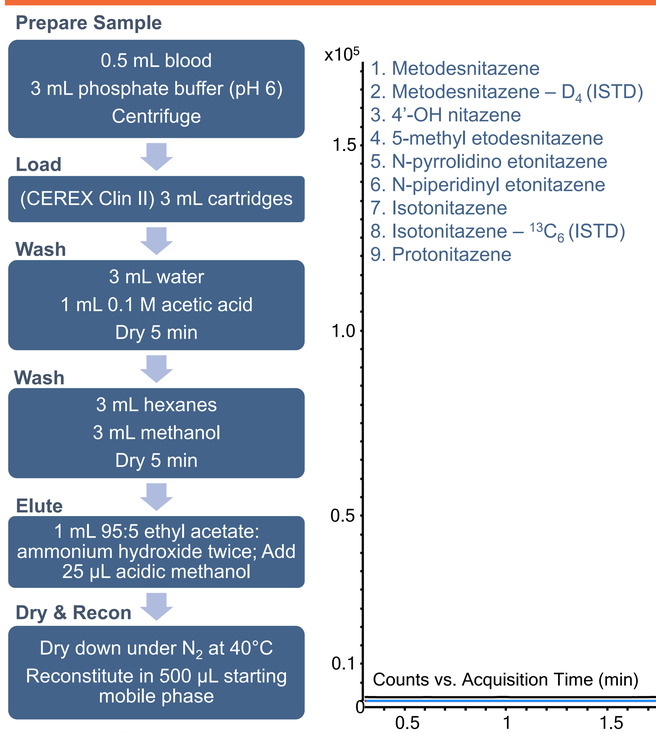

Figure 2. *SPE protocol*

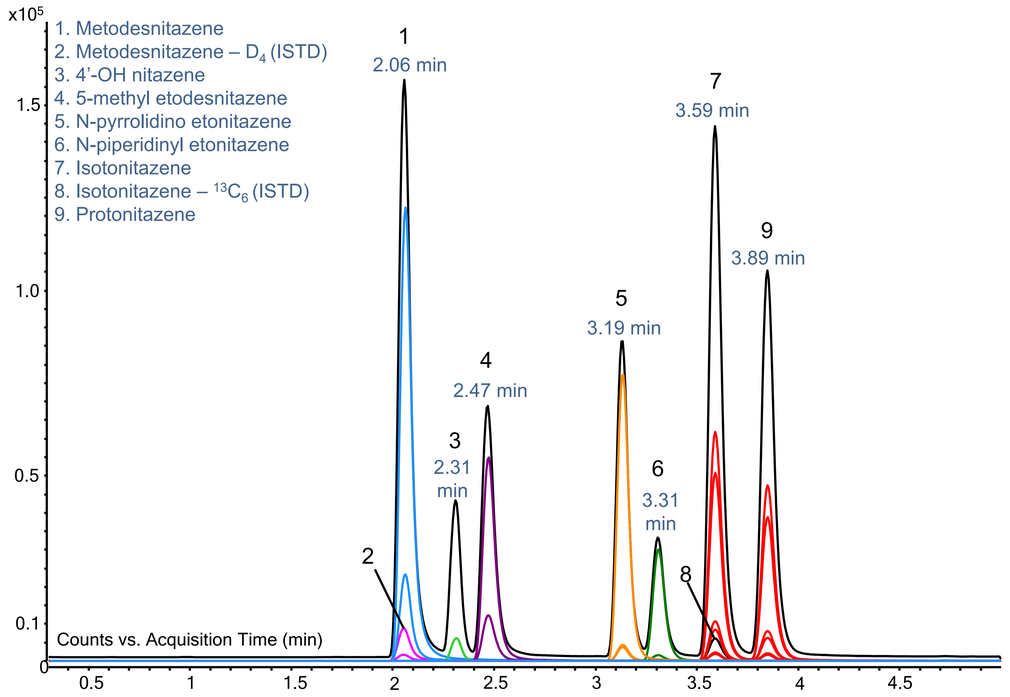

Figure 3. *Example chromatogram for a 25 ng/mL extracted sample*

Table 4. *Key validation results*

Parameter	Results
Calibration Model	Quadratic 1/x 5-methyl etodesnitazene, linear 1/x all other analytes; 0.5 – 100 ng/mL; R^2>0.991
Limit of Detection	0.25 ng/mL - metodesnitazene, isotonitazene, protonitazene 0.5 ng/mL - 4'-OH nitazene, 5-methyl etodesnitazene, N-piperidinyl etonitazene, N-pyrrolidino etonitazene
Lower Limit of Quantification	0.5 ng/mL
Within- and Between-run precision (%CV)	Between run precision within 17% Within run precision within 22%
Grand Bias (%)	Within ±19%
Matrix Effects (%)	Acceptable ion suppression/enhancement except metodesnitazene-D4 (>25% suppression); Additional sources of matrix used for LOD/LLOQ *Human and bovine blood sources comparable*
Carryover	<LOD
Interferences	No qualitative interferences
Processed Stability (72 h)	All analytes stable

MATERIALS & METHODS

LC-MS/MS method

Analysis was completed with an Agilent 1290 Infinity II Liquid Chromatograph and an Agilent 6470 Triple Quadrupole Mass Spectrometer operated in positive electrospray ionization mode with optimized instrumental conditions (**Table 2**). Data were acquired using MRM and optimal transitions (**Table 3**).

Table 3. *Optimized instrumental parameters*

Parameter	Value
Column	Agilent Poroshell 120 EC-C18 column (2.1 x 100 mm x 2.7 µm) with matching guard
Mobile phase A	0.1% formic acid and 5 mM ammonium formate in water
Mobile phase B	0.1% formic acid in acetonitrile
Column temp	35 ºC
Flow rate	0.4 mL/min
Gradient	10% B, until t=0.25 min., ramped up until 25% B at t=1 min., then 35% B at t=1.75 min, then 50% B at 4.75 min; 2 min 90% B wash; 2 min. re-equilibration; total run time 9 min.
Injection volume	5 µL

Electrospray ionization parameters were as follows: Drying gas temperature 350 ºC, gas flow 9 L/min, nebulizer 35 psi, sheath gas temperature 400 ºC, sheath gas flow 12 L/min, capillary (+) voltage 4000 V, and nozzle (+) voltage 0 V.

Table 2. *MRM transitions for each analyte (quantifier ions are bolded)*

Compound	Precursor ion (*m/z*)	Product ions (*m/z*)
Isotonitazene	411	107, **100.1**, 72.1
Isotonitazene-$^{13}C_6$	417	107, **100.1**, 72.1
Metodesnitazene	338	**100.1**, 72.1
Metodesnitazene-D_4	342	**104.1**, 76.1
N-pyrrolidino etonitazene	395.2	107, **98.1**, 77.1
Protonitazene	411	107, **100.1**, 72.1
N-piperidinyl etonitazene	409	**112**, 77
5-methyl etodesnitazene	366	**100.1**, 72.1
4-OH nitazene	369	**100.1**, 72.1

Sample Preparation & Method Validation

Calibrators and controls were prepared by fortifying whole bovine blood with analyte and internal standard. A solid-phase extraction procedure was developed by adapting a method from the literature [2] using 0.5 mL sample (**Figure 2**). Method validation was performed with guidance from ASB 036 [3]. Method performance was evaluated with both bovine and human whole blood matrix sources.

CONCLUSIONS

- A method was developed and validated for the quantitation of seven nitazenes in whole blood.
- Of note, some of the newer, currently prevalent nitazenes including 5-methyl etodesnitazene are included in the panel.
- A comparison of performance between bovine and human blood supported the use of bovine blood for preparation of calibrators and controls.
- The method utilizes instruments and reagents common in forensic toxicology laboratories.
- The working range of 0.5 – 100 ng/mL and LODs of 0.25 - 0.5 ng/mL is consistent with methods described in literature for nitazene detection using LC-MS/MS, demonstrating the utility of this method for forensic toxicology casework.